A SURVEY OF PURINE DERIVATIVES AS INDUCERS OF CHROMOSOME CHANGES

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FIL. LIC., VG.

INAUGURAL DISSERTATION

FOR THE DEGREE OF DOCTOR OF PHILOSOPHY WHICH BY DUE PERMISSION OF THE PHILOSOPHICAL FACULTY OF THE UNIVERSITY OF UPPSALA WILL BE PUBLICLY DEFENDED IN THE BOTANICAL AUDITORIUM, ON MAY 24th, 1952, AT 10:00 A. M.

LUND 1952, BERLINGSKA BOKTRYCKERIET

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BY *BENGT KIHLMAN*

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I. MUTAGENIC EFFECT ON MICRO-ORGANISMS.

THAT mutations can be induced by caffeine and theophylline was shown by FRIES and KIHLMAN (1948). They found a considerably higher percentage of biochemical mutants in conidial cultures of the ascomycete *Ophiostoma multiannulatum* treated with these purine derivatives than in untreated material. Thus, after a 5-days treatment with 0,2 % caffeine, 0,8 % mutant conidia was obtained, while untreated conidial cultures were found to contain never more than 0,06 %. A 4-days treatment with 0,6 % theophylline resulted in 0,6 % mutant conidia.

FRIES (1950) showed that the increased percentage of mutants resulting from caffeine treatments cannot be explained merely as a result of a selection, through differential killing, of mutants already existing in the conidial culture.

Independently of the experiments of FRIES and KIHLMAN, WITKIN demonstrated that caffeine is able to induce mutations to phage resistance in the bacterium *Escherichia coli* (quoted from DEMEREC, 1949), results which have been confirmed recently by GEZELIUS and FRIES (1952). DEMEREC, BERTANI and FLINT (1951) have found that caffeine is able to induce back-mutations from streptomycin dependence to non-dependence in *E. coli*.

II. RADIOMIMETIC EFFECT ON HIGHER PLANTS.

1. INVESTIGATIONS ON ALLIUM CEPA.

Since mutagenic substances in general are able to induce structural chromosome changes, caffeine was also tested in cytological experiments. As a result of the experiments of GOSSELIN (1940) and DEYSSON (1948), *etc.*, it is known that caffeine (1) inhibits cell-wall formation after mitosis so that binuclear and later multinuclear cells are produced, and (2) induces bridge formation at anaphase.

In the investigations of KIHLMAN and LEVAN (1949), root tips of

common onion, *Allium cepa*, were used as experimental material. The experiments showed that solutions containing more than 0,04 % of caffeine suppress mitosis, a total suppression being obtained with 0,4 % caffeine. In concentrations stronger than 0,02 % caffeine, as well as theophylline and theobromine, suppresses cell-wall formation. The methylxanthines mentioned do not inhibit spindle function, *i. e.*, they do not induce full c-mitosis.

Of particular interest in this connection are the effects on the chromosomes themselves. Using caffeine in concentrations above 0,04 %, chromosome changes were observed within 1 hr. after commencement of the treatment. These changes were mostly of the type due to stickiness of the chromosome matrix, *i. e.*, clumping of the chromosomes at metaphase, as well as formation of chromatin bridges between separating chromosome arms at anaphase. Another change appearing soon after the beginning of the treatment is the so-called pseudo-chiasma phenomenon (LEVAN, 1949).

The formation of anaphase bridges after caffeine treatment, as observed by DEYSSON, was probably due to primary effects of the types just described. KIHLMAN and LEVAN found, however, that after caffeine treatments a secondary effect is also demonstrable. [The terms »primary» and »secondary» effect were originated by ALBERTI and POLITZER (1923). According to these workers, the »Primäreffekt» is exhibited by cells already in division at the time of treatment; while the »Sekundäreffekt» is observable in cells entering mitosis after the end of the »mitosenfreie Zwischenzeit», which follows the treatment. Secondary effects are thus induced in resting cells.] Secondary effects were studied in root tips which had been treated for 24 hours in solutions containing 0,1—0,4 % caffeine and afterwards allowed to recover in water, the recovery period being 1—7 days. The changes observed were of the usual type known from X-ray experiments and treatments with mustards, *e. g.*, fragmentations and chromosome bridges at anaphase. The bridges expose most of the dicentric chromatids which have arisen as a result of sister chromatid union in centric fragments at the point of breakage, or as a result of reciprocal translocation. After the recovery period, the chromosomes showed no sign of stickiness.

The results of KIHLMAN and LEVAN were later confirmed qualitatively by LOVELESS and REVELL (1949).

Thus it was found that caffeine, in addition to having a mutagenic effect on micro-organisms, in cytological experiments is able to induce chromosome changes of the same kind as are obtained after X-ray

irradiation, *i. e.*, it has a *radiomimetic* effect. The question now arises: Upon what chemical and physical properties of the caffeine molecule does its ability to induce chromosome changes depend? By testing the effects of a great number of compounds closely related to caffeine, it should perhaps be possible to answer this question. The following purines and related compounds were therefore synthesized: 1,7-dimethylhypoxanthine (DMH), 8-chlorocaffeine (CC), 8-methoxycaffeine (MOC), 8-ethoxycaffeine (EOC), 8-propyloxycaffeine (POC), 8-allyloxycaffeine (AOC), 8-methylthiocaffeine (MTC), 8-ethylthiocaffeine (ETC), 1,3,7-trimethyluric acid or 8-hydroxycaffeine (HOC), 1,3,7,9-tetramethyluric acid (TMU), 9-ethyl-1,3,7-trimethyluric acid (ETMU), 9-allyl-1,3,7-trimethyluric acid (ATMU), and 1,3,7,9-tetramethyl-4,5-dimethoxydihydrouric acid (TDDU). From other sources the following were obtained: hypoxanthine, xanthine, uric acid, adenine, guanine, theophylline, theobromine, 8-methylaminocaffeine (MAC), 1,3-diethylxanthine (DEX), 1,3,7-triethylxanthine (TEX), and 8-hydroxy-9-methyl-8,9-dihydrocaffeine (HMDC). The effects of these 24 compounds on the root-tip chromosomes of *Allium cepa* were thereupon tested. Detailed descriptions and discussions of the method used and the results obtained have recently been published (KIHLMAN, 1951, 1952). The main results are summarized below.

Toxicity. — The purine derivatives in general have a lethal effect only in high concentrations. If in methylated oxypurines, the methyl groups are substituted by ethyl or other alkyl groups with more carbon atoms in the chain, the toxicity is increased. Thus, DEX and TEX are considerably more toxic than the corresponding methylxanthines, *viz.*, theophylline and caffeine. This rule is valid not only for nitrogen-bound alkyl groups but for alkoxy groups as well. The toxicity is further correlated with the temperature during the period of treatment; the higher the temperature, the greater being the toxicity.

Inhibition of spindle function. — In general purine derivatives do not induce full c-mitosis. Exceptions are the ethylxanthines and adenine. The former compounds obey the rule formulated by LEVAN and ÖSTERGREN (1943), *viz.*, that the lower the water solubility of the substances, the lower their threshold concentrations for c-mitotic action. (The decisive factor in c-mitosis, however, should not be the water solubility of the substances but their relative lipoid solubility, which was assumed to be inversely proportional to the water solubility.) However, of the purines tested, adenine has both the most pronounced c-mitotic action and the lowest relative lipoid solubility. It would seem, therefore,

that the c-mitotic action of adenine, like that of colchicine, may be due to a more specific reaction than that connected with relative lipoid solubility.

The failure of cell-wall formation. — The ability to suppress cell-wall formation seems to be a property common to all N-alkylated oxypurines. It is, however, seldom so pronounced as after treatments with caffeine. A strong effect is also observed after treatments with MTC. As already mentioned, the suppression of cell-wall formation results in the origin of bi- and multinuclear cells. In such cells the nuclei may fuse during mitosis producing cells with a high number of chromosomes. Polyploid cells may thus arise as a result of two different mechanisms after treatment with purine derivatives, namely (1) c-mitosis (adenine, ethylxanthines) and (2) suppression of cell-wall formation (N-alkylated oxypurines).

Effects on chromosomes. — In general, the chromosome changes observed are of the same type as those already described in connection with the caffeine experiments, though from a quantitative point of view the purines tested behaved very differently. Some of them were totally or practically inactive, while others had an activity many times greater than that of caffeine. The most pronounced stickiness effect was obtained after treatments with the 8-ethers and 8-thioethers of caffeine, but also CC and TMU were active in this respect. Pseudo-chiasmata were particularly common after treatments with MAC.

In order to compare the chromosome-breaking abilities of the purines, their effects were calculated on a molar basis. The purines were classified as (1) very active, (2) active, (3) slightly active, and (4) inactive. The »very active» type comprised all the 8-substituted caffeine derivatives tested, HOC excepted. A marked effect was also obtained with TMU. In the *Allium* experiments, all the non-alkylated oxypurines were inactive, as were also DMH, the ethylxanthines, HOC, TDDU, and HMDC. Adenine, in high concentrations (40—20mmol/l.), possessed a rather marked chromosome-breaking ability; however, calculated on a molar basis, its effect is slight compared with that of the 8-substituted caffeine derivatives. After adenine treatments, such disturbances as »attached fragments» and »chromosome erosion» (Levan and Tjio, 1948) were unusually frequent.

The following relations were found between the radiomimetic effect of purines and their chemical constitution:

(1) The radiomimetic activity of oxypurines increases with increasing N-methylation.

(2) The activity of methylated oxypurines diminishes or is completely lost if methyl groups are substituted with groups possessing longer carbon chains. (In this respect the radiomimetic effect is inversely proportional to the toxicity.)

(3) In caffeine, substitution of the hydrogen atom at C_8 with an electro-negative substituent, generally results in an increase in activity.

(4) The inability of HOC to induce chromosome changes indicates that the introduction of an acid, ionizable group in the molecule destroys the radiomimetic activity.

Further, it seems as if the double bond adjacent to C_8 or the 4—5 double bond should be of significance with regard to radiomimetic activity.

Chromosome breaks and rearrangements, as well as stickiness and pseudo-chiasmata, are induced by purines only above a certain threshold concentration which differs for different purines. This threshold is considerably higher (up to ten times) for the former type of changes than for the latter. Pseudo-chiasmata may be induced by more dilute solutions than may stickiness, and the threshold is thus lowest for this type of chromosome change.

It was also found that the active purine derivatives did not behave similarly in the experiments. Two types could be distinguished: The first is represented by ETC and the 8-ethers of caffeine, with the exception of MOC. These purines act rapidly, so that after a few hours most of the treated cells showed chromosome breaks. The effect increases in proportion to the concentration and is independent of the mitotic activity during the period of treatment. This mode of action is clearly demonstrated in the effect of EOC. To the EOC group belong such purines as have the same mode of action.

With the other type of active purine derivatives, chromosome breaks are obtained in a large number of cells only after a long period of treatment (about two days), and in concentrations sufficiently low to permit a certain amount of mitotic activity during treatment. After treatment with concentrations suppressing mitosis completely, the percentage of abnormal cells is always low. Thus, it is evident that the percentage of abnormal cells, obtained after treatment with purines of this group (the TMU group), is dependent on the mitotic activity during the treatment-period; the more mitoses occurring during the treatment, the greater the number of abnormal cells. When the effect of this group of purines is expressed in terms of the number of breaks per abnormal

cell, however, it shows the same dose proportionality as does the effect of the purines belonging to the EOC group.

The effect of purine derivatives on chromosomes is more or less independent of the *pH* of the treatment solution. On the other hand, the *temperature* during the period of treatment is of great significance. It is of interest to note that the temperature influences the effect of EOC and TMU in two different ways. With EOC, the strongest effect is obtained when the temperature during treatment is around 10° C. The effect at 30° C is only three quarters of that at 10°C. With TMU, however, only a low number of abnormal cells is obtained at 10° C, this purine being most effective at temperatures around 20° C. The explanation is that at 10° C the mitotic activity is low, and since the radiomimetic effect of TMU, if expressed in percentage of abnormal cells, is dependent on mitotic activity, it must also be low.

2. INVESTIGATIONS ON PISUM SATIVUM.

Some of the purine derivatives were also tested in experiments with *Pisum sativum* (KIHLMAN, 1952). The same types of chromosome changes and the same quantitative differences in activity between the purines, as previously observed in the *Allium* experiments, were found. The root-tip chromosomes of *Pisum*, however, are much less susceptible to the radiomimetic effects of the purines than are those of *Allium*, this rule applying to the primary as well as to the secondary effect. The characteristic difference between EOC and TMU as regards their mode of action did not appear so clear-cut in the *Pisum* experiments as in *Allium*, the reason being that in *Pisum*, TMU does not induce chromosome breaks at concentrations such as do not inhibit mitosis.

Particular attention was paid to the effect of adenine. In high concentrations (40—20 mmol/l.), this purine induces, as in *Allium*, chromosome breaks and erosion. In concentrations $\geqq 5$ mmol/l., adenine totally inhibits the function of the spindle and/or that of the centromere, and produces a pronounced chromosome contraction. Most mitoses, however, seem to be arrested at late prophase and in this respect the effect of adenine differs from that of colchicine. After treatment at concentrations just below the threshold for the c-mitotic action, lag chromatids were frequently observed. Ana- and telophase bridges were found after treatments with solutions containing 2,5 mmol/l. of adenine. These bridges seem to have arisen through a stretching of the centromeres of lagging chromatids in the direction of the spindle axis.

The cytological effects of purines have recently been studied in

tissue cultures (mouse-embryo skin, sarcoma), by BIESELE (1951). He found, *i. a.*, that 2,6-diaminopurine caused chromosome breakage in low concentrations and that adenine in higher concentrations was very effective.

Analyses of the localization of the chromosome breaks were also performed. Previously, KIHLMAN and LEVAN (1951) found that if root-tip chromosomes of *Vicia faba* were treated for 6 hours at 10° C with 0.2 % of EOC, most of the breaks induced were localized to the attachment thread of the satellite. When the treatment was prolonged, breaks in the chromosomes were also noticed at other points. A less marked localization of breaks to the satellite fibre was found after TMU treatments.

In the *Pisum* investigations, satellite fragments were found after treatments with the active purines tested, with the possible exception of adenine. Of the total number of fragments recorded, however, the percentage of satellite fragments was considerably lower than in the *Vicia* experiments, seldom exceeding 50.

In addition to the satellite fibre, breaks seemed to be localized to a region close to the centromere in the chromosomes. In order to analyse the distribution of breaks in greater detail, it was necessary to determine the morphology of the *Pisum* chromosomes. With the aid of the oxyquinoline technique of TJIO and LEVAN (1950), the seven chromosome pairs of *Pisum sativum* were identified and characterized. It appeared that the secondary constriction of one of the chromosomes differs from the secondary constrictions of others in three ways: (1) by its length, (2) by its failure to contract during treatment with oxyquinoline, and (3) by the probability of its being in contact with the nucleolus during prophase. This »secondary constriction» chromosome is called the satellite chromosome although, according to HÅKANSSON and LEVAN (1942), it has nothing to do with the formation of the nucleolus.

By means of the oxyquinoline technique, a detailed analysis was made of the distribution of breaks not lying in the satellite fibre. In this analysis, only those cells were considered in which most or all of the chromosomes could be identified, a total of 50 breaks in 40 cells being analysed. 28 of these breaks, *i. e.*, 56 %, were localized close to a centromere. As regards the distribution of the breaks on different chromosomes, it was found that, in particular, the satellite chromosome and the largest chromosome were frequently broken. Including the breaks in the satellite fibre, considerably more than the half of the EOC-induced breaks were localized to the satellite chromosome.

Finally, a comparison was made between the radiomimetic effect of EOC, nitrogen mustard and maleic hydrazide on *Pisum*. It may be mentioned that the same number of breaks (about one break per cell) as induced by EOC in a concentration of 7,5 mmol/l., was induced by maleic hydrazide in a concentration of 0,2 mmol/l. and by nitrogen mustard in a concentration of only 0,01 mmol/l. EOC, however, has the advantage over nitrogen mustard in that it has a low toxicity. From a qualitative point of view, the effect of EOC differs considerably from that of maleic hydrazide.

III. THE MODE OF ACTION OF PURINE DERIVATIVES.

1. THE ENTRANCE INTO THE NUCLEUS.

The entrance into the cell probably presents no difficulties for purine derivatives, at least for those with an almost neutral reaction. The passage into the nucleus, however, seems to be blocked rather effectively. The simplest explanation of the difference in the mode of action between purines of the TMU and EOC type described above, is that only the latter are able to penetrate the nuclear membrane, so that the concentration inside the nucleus exceeds the threshold for the radiomimetic action. Purines of the TMU type can enter the nucleus only during mitosis, when the nuclear membrane disappears. This should explain why the radiomimetic effect of these purines, expressed in per cent of abnormal cells, is dependent upon the number of mitoses during the period of treatment.

This hypothesis is supported by determinations of the relative lipoid solubilities of the purines, the results of these investigations showing that the hypothesis is consistent with the lipoid theory of Overton (1899, 1902). It appeared from determinations of the distribution coefficients in the systems ethyl ether/water and oleyl alcohol/water, that purines of the EOC type have a stronger affinity for the »lipoid» phase, while purines of the TMU type have a stronger affinity for the water phase of the system. Only those purines having a stronger affinity for the »lipoid» phase seem to be able to penetrate the nuclear envelope. This implies, however, that the nucleus is surrounded by a lipoid membrane. Such a membrane has been demonstrated by several workers in different organisms and by different methods. In *Allium*, the present author obtained evidence for the existence of a perinuclear lipoid layer by means of staining tests with the lipoid stain Sudan black B. Further evidence was obtained by Berg (1951), who found, by

means of his benzpyrene-caffeine method, a well-designed lipoid membrane around nuclei of onion root-tip cells.

It may seem somewhat surprising that a compound which easily penetrates the cell membrane, is practically unable to penetrate the nuclear envelope. Such strong barriers of lipoidic nature as are assumed to protect the nuclei of *Allium* root-tip cells are, however, not unknown in physiology, an interesting case having been described by NACHMANSOHN (1950).

Physostigmine and di-isopropylfluorophosphate (DFP) abolish nerve conduction. This effects is due to the inhibitory action of these drugs on acetylcholine esterase, an enzyme involved in nerve function. Prostigmine, a compound related to physostigmine, and tetraethylpyrophosphate (TEPP) are extremely potent inhibitors of acetylcholine esterase, but do not affect nerve conduction. The explanation seems to be that physostigmine and DFP, in contrast to prostigmine and TEPP, are able to penetrate into the axoplasm. The former compounds are lipoid soluble, the latter not. The structural barrier, which prevents prostigmine and TEPP from penetrating into the axoplasm and thus from coming in contact with the enzyme, is, according to NACHMANSOHN, probably the lipoid membrane, known to surround cell axons.

Since methylxanthines also are inhibitors of acetylcholine esterase, it should be of interest to know whether purines of the EOC type are able to penetrate the structural barrier surrounding axons and to affect nerve conduction.

2. BEHAVIOUR INSIDE THE NUCLEUS.

After the entrance into the nucleus, the purine derivatives, or at least those with surface activity, probably accumulate on intra-nuclear surfaces such as that of the nucleolus or the inner surface of the nuclear membrane. On these surfaces, the purines would at first exceed their threshold concentrations for chromosome-breaking ability, and those chromosome parts in contact with these surfaces would be broken first. This would explain why the breaks, induced in *Vicia faba* chromosomes by EOC, are localized to the satellite fibre, since this chromosome region is in contact with the surface of the nucleolus during interphase and prophase. Another explanation of the satellite breaks could be that the satellite fibres, and possibly secondary constrictions in general, have a greater affinity for purines or are more susceptible to their radiomimetic effects than are other parts of the chromosomes. It was hoped that the *Pisum* investigations should throw light upon the question, since, according to HÅKANSSON and LEVAN (1942), the satellite chromosomes in *Pisum sativum* have nothing to do with the formation and

localization of the nucleolus. Instead, nucleoli are formed at telophase at the centromeric regions of other chromosomes. These primary nucleoli fuse into one large nucleolus, which during interphase is attached to the centromeric regions of one or two chromosomes.

If, after EOC treatments, only a few breaks occur in the *Pisum* satellite fibre, and particularly if the breaks are localized to the centromeric region, it would seem to indicate that this localization is connected in some way or other with the nucleolus. As we have seen, most breaks were localized to the satellite fibre or to regions close to the centromeres. These results were at first rather confusing, but when later it was found that the satellites, at least during prophase, as a rule appear to lie on the surface of the nucleolus, the localization of breaks observed could be explained on the basis of the »nucleolus hypothesis». However, the results of the *Pisum* investigations are not entirely convincing, and until more evidence has been produced in support of this hypothesis, the other explanations of satellite breaks already mentioned cannot be excluded.

The »nucleolus hypothesis» accounting for the localization of breaks is based on the assumption that EOC and other purines having a similar effect, accumulate on surfaces. Also, regarding radiomimetic activity in general, a surface or boundary activity may be significant, since it is possible that the purine derivatives must be adsorbed on or come in contact with the chromosomes before breaks can be induced. For these reasons it should be of interest to obtain at least an approximative idea of the boundary activity of the purines.

As a measure of the boundary activity, a determination was made of the power of purines to replace methylene blue on the surface of charcoal (KIHLMAN, 1952). The radiomimetic purine derivatives showed a considerable boundary activity. It was found that all the purines which are »very active» inducers of chromosome breaks also have a high boundary activity and that those purines which are »inactive» or only »slightly active» in cytological tests have in general a low boundary activity. The most striking difference between the boundary activity of purines and their radiomimetic activity is that the former increases if N-bound methyl groups are substituted by groups with longer carbon chains while the latter decreases. The reason for this difference may be that a specific adsorption is involved. A specific adsorption on an intra-nuclear enzyme in competition with its substrate or co-enzyme (for instance, adenine, guanine or compounds containing these purines as constituents) could be the cause of the radiomimetic activity of

purine derivatives. It is also possible that the radiomimetic effect is favoured only by a high boundary activity, but that the chromosome-breaking ability itself is dependent upon other properties of the purine molecule, which could be altered by the lengthening of the carbon chains in N-bound alkyl groups. But if this is so, which properties could be taken into consideration?

The experiments have shown that the radiomimetic activity of purines is not dependent on any particular atomic group but on the molecule as a whole. Chemically, the purines are rather inactive and it is difficult to imagine that they should be able to break chemical bonds by a direct reaction. However, since chromosome breaks are probably breaks between molecules (see MULLER, 1941) and since the forces operating between molecules are of another character and considerably weaker than the very strong bonds between atoms, a direct action of purine derivatives cannot be excluded.

A striking correlation has been found between the radiomimetic activity of purines and their solubilizing power (WEIL-MALHERBE, 1946). Therefore, one could assume that the radiomimetic effect is a direct result of a solubilizing action of purines on the genetic material. Investigations of the solvent power of purines using, as solubilizates, the dye Congo rubin and desoxyribonucleohistone, indicates, however, that the radiomimetic effect cannot be explained in this way (KIHLMAN, 1952). Among other things, it was found that the solubilizing power — in contrast to the radiomimetic effect — does not disappear when N-bound carbon chains are lengthened, although a certain decrease could be observed. Thus, the solvent power cannot account for the difference in the radiomimetic effect found between the N-methylated purine derivatives and their corresponding higher homologues.

On the other hand, the good correlation between solvent power and radiomimetic activity of purines cannot be due to mere chance, however. According to WEIL-MALHERBE, the solvent power of purines is dependent upon the same intermolecular attraction forces which are responsible for the formation of molecular compounds. It is concluded that these »intermolecular attraction forces» also play a significant rôle in the radiomimetic activity of purines.

This does not necessarily mean that the purines act directly on intermolecular bonds existing in chromosomes. A competitive inhibition of enzymes is probably dependent upon molecular attraction between enzyme protein and inhibitor substance. Purines may also inactivate enzymes by forming molecular complexes with their prosthetic

groups or co-enzymes. According to BURTON (1951), the inhibitory action of caffeine on d-amino acid oxidase is due to the formation of a non-fluorescent complex between caffeine and the prosthetic group, in this case adenine flavine dinucleotide.

Unfortunately, very little is known about the effects of purine derivatives on intra-nuclear enzyme systems. In one experiment a test was made of the effect of EOC and TMU on the desoxyribonucleic acid phosphatase complex in nuclei (KIHLMAN, 1952). The activity of the enzyme was determined according to the histochemical method worked out by GOMORI (1946). However, these purines appeared to have no effect upon phosphatase activity.

Future investigations on the mode of action of purine derivatives on chromosomes are planned by the author.

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